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FINAL TECHNICAL REPORT SUBMITTED TO THE OFFICE OF NAVAL RESEARCH FOR PROJECT ONR N00014-87-K-0378

Entitled

"Strategies for Biopolymer Engineering of PHB-Like Materials"

Department of Biology and Laboratory of Biomaterials Science & Engineering,
Massachusetts Institute of Technology, Cambridge, MA. 02139

Submitted by

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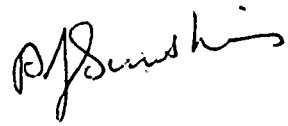

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INTRODUCTION

As part of renewal application for Grant ONR N00014-87-K-0378, a final technical report was included in the application. This report is extracted from the progress report submitted to the Office of Naval Research Code 1141, Director of Biological Sciences Division, 800 N. Quincy Street, Arlington, Virginia 22217-5000. Also included in the renewal application was an appendix containing a list of all technical publications resulting from the project. A revised publication list is included with this report since in 1989 several publications were submitted or in press

In addition, patent applications and continuation based on this work have been filed through the MIT licensing office and are listed as Appendix II.

Final Technical Progress Report for Grant ONR-N00014-87-K-038

Our initial studies on PHB biosynthesis focussed on a mechanistic analysis of the first enzyme in the pathway, ß-ketothiolase, from Z. ramigera (Davis et al., 1987a; 1987b; Peoples et al., 1987a). Using classic inhibitor studies coupled with site-directed mutagenesis, we have proven that the biosynthetic thiolase proceeds through a two step mechanism. In the first half reaction, the enzyme's active site, Cys89, attacks acetyl-CoA to form an acetyl-S-enzyme intermediate (Davis et al., 1987a; Walsh et al., 1989; Thompson et al., 1989; Masamune et al., 1989b) and in the second half of the reaction, this intermediate reacts with the anion formed from the second acetyl-CoA molecule by enzymatic deprotonation to complete the condensation. The enzyme's catalytic base involved in extracting the proton from the second acetyl-CoA to form the anion has been identified as Cys378 (Masamune et al., 1989a,b; Davis et al., 1989; Differding et al., 1989; Palmer, M. et al.,

1989). Both active site residues are conserved in all the thiolase sequences reported to date (Peoples and Sinskey, 1989b, see Appendix 1; Submitted to ONR August 1989).

Following the cloning and sequencing of the Z. ramigera thiolase gene, the NADPH-specific acetoacetyl-CoA reductase gene was identified downstream (Peoples and Sinskey, 1989a, see Appendix 1 submitted to ONR August 1989) and the overproduced enzyme purified from E. coli and studied for substrate utilization (Ploux et al., 1988). DNA hybridization experiments, using the Z. ramigera genes, enabled us to isolate the corresponding two genes from the industrially more important bacterium A. eutrophus (Peoples and Sinskey, 1987; Peoples and Sinskey, 1989b, see Appendix 1; Submitted to ONR August 1989). Characterization of both thiolases and reductases from E. coli overproducers indicated that these same enzymes are most likely responsible for the synthesis of not only (D)-ß-hydroxybutyryl-CoA but also (D)-ß-hydroxyvaleryl-CoA as monomers for the synthesis of the PHB-PHV copolymers. Both the thiolase and reductase enzymes also can utilize ß-ketohexanoyl-CoA although only at around 0.1% the activity of the C4 substrate.

Located immediately upstream from the A. eutrophus thiolase gene is the PHB polymerase gene (Peoples and Sinskey, 1989c, see Appendix 1). Hence, in this bacterium, the three genes of the PHB biosynthetic pathway are organized as phbC-phbA-phbB, as illustrated in Figure 1. The genes encode PHB polymerase, ß-ketothiolase and NADPH-specific acetoacetyl-CoA reductase respectively. Expression of all three genes from a promoter located upstream from phbC is required to obtain the production of significant (up to 50% dry cell weight) levels of PHB in E. coli (Peoples and Sinskey, 1989c). The predicted translation product of the PHB polymerase gene is a protein of Mr=63900 that has a hydropathy profile distinct from typical membrane spanning

proteins implying that PHB biosynthesis may not require a membrane. These data agree with recent NMR studies on intact PHB granules from Methylobacterium and A. eutrophus (Barnard and Sanders, 1988; 1989).

In order to use the capacity of P. oleovorans for polymerizing longer chain length (D)-hydroxyacyl-CoA substrates in the design and engineering of novel PHA production systems, we isolated the PHA polymerase gene using the A. eutrophus phbC gene as a hybridization probe. Plasmid pPO23 contains a 6.4kb EcoR1 restriction fragment of P. oleovarans chromosomal DNA and the complete nucleotide sequence was determined. Figure 2 illustrates the locations of the open reading frames present on this insert. ORF1 encodes a polypeptide of 562 amino acids with an Mr=60000 and having 52% sequence identity with the A. eutrophus PHB polymerase identifying this gene as PHA polymerase. The identities of the proteins encoded by the two ORF's located downstream are not known at this time.

Conclusion

The above results now allow for us to focus on completing the characterization of the A. eutrophus PHB biosynthetic genes by identifying the sequences responsible for expression of phbC-phbA-phbB and studying the regulation of these genes in this system. In conjunction with these experiments, we are now monitoring the fate of the polymerase protein using antibodies raised against the enzyme purified from an E. coli overproducer. Our studies on the P. oleovarans system have focused initially on completing the characterization of the genes including the identification of the products of ORF's 2 and 3. Overproduction of both polymerase enzymes in parallel will enable us to study their substrate specificities and undertake in vitro PHA biosynthesis. From these experiments, we may be able to design hybrid

polymerase genes with intermediate specificity. The potential for regulating the expression of these genes to control or produce novel polymers in homologous and heterologous systems will be investigated. A detailed characterization of the physical/rheological properties of the materials produced will be undertaken.

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Davis, J.T., M.A.J. Palmer, O.P. Peoples, S. Masamune, A.J. Sinskey and C.T. Walsh. 1939. Biosynthetic thiolases from *Zoogloea ramigera* and *A. eutrophus*: The inactivation with 5,5'-dithiobis92-nitrobenzoate) and diethyl azodicarboxylate. Submitted to Biochemistry.

Davis, J.T., R.N. Moore, B. Imperiali, A. Pratt, K. Kobayashi, S. Masamune, A.J. Sinskey and C.T. Walsh. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. 1. Preliminary characterization and analysis of proton transfer reaction. J. Biol. Chem. 262:82-89.

Differding, E., R. Gamboni, S.F. Williams, M.A.J. Palmer, O.P. Peoples, S. Masamune, A.J. Sinskey and C.T. Walsh. 1989. Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera*: Inhibition with 3-pentynoyl-pantetheine-11-pivalate. Submitted to Biochemistry.

Masamune, S., M.A.J. Palmer, R. Gamboni, S. Thompson, J.T. Davis, S.F. Williams, O.P. Peoples, A.J. Sinskey and C.T. Walsh. 1989. Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera*. Active site cysteine residues. J. Amer. Chem. Soc. 111:1879-1881.

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Palmer, M.A.J., Gamboni, R., Williams, S.F., Peoples, O.P., Masamune, S., Sinskey, A.J. and Walsh, C.T. 1989. Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera* inactivation with acryl-S-pantetheine-11-pivalate and characterization of the Gly-378 mutant thiolase enzyme. Submitted to Biochemistry.

Peoples, O.P. and Sinskey, A.J. 1987. Polyhydroxybutyrate (PHB): A model system for biopolymer engineering. Prog. Biotechnol. 3:51-56.

Peoples, O.P. and Sinskey, A.J. 1989a. Fine structural analysis of the *Zoogloea ramigera* *phbA-phbB* locus encoding ß-ketothiolase and acetoacetyl-coa reductase: nucleoside sequence of *phbB*. Molecular Microbiology 3:349-357.

Peoples, O.P. and Sinskey, A.J. 1989b. Poly(B)-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16: Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:

Peoples, O.P. and Sinskey, A.J. 1989c. Poly-ß-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Characterization of the genes encoding ß-ketothiolase and acetoacetyl-coa reductase. J. Biol. Chem. 264:

Thompson, S., F. Mayer, O.P. Peoples, S. Masamune, A.J. Sinskey and C.T. Walsh. 1989. Mechanistic studies on ß-ketoacyl thiolase from Z. ramigera: Identification of the active-site nucleophile as Cys_{89}, its mutation to Ser_{89}, and kinetic and thermodynamic characterization of wild type and mutant enzymes. Biochemistry, 28:5735-5742.

Walsh, C., W.H. Chen, E. Differding, S. Masamune, O. Peoples, O. Ploux, A.J. Sinskey and S. Thompson. 1989. Enzymes in the biosynthesis of polyesters. Proc. Robert A. Walsh Found. Conf. Chem. Res. 31:184-203.

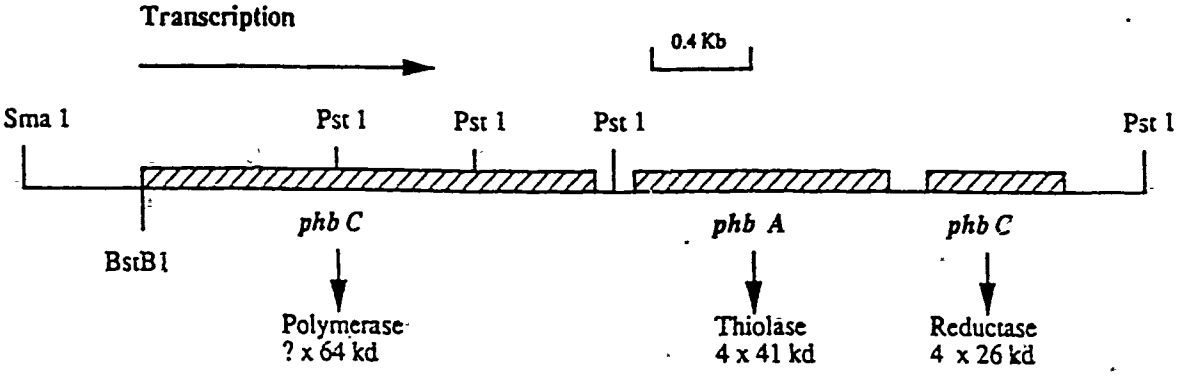

Figure 1 Organization of the Alcaligenes eutrophus phbC-phbA-phbB genes.*

* Peoples & Sinskey 1989c.

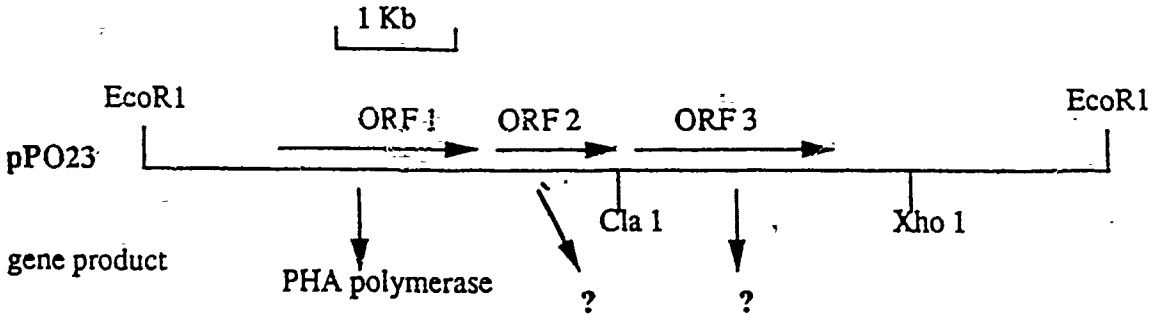

Figure 2. Organization of the Pseudomonas oleovarans PHA polymerase locus.*

*Peoples & Sinskey 1989 unpublished. (Note that PHA polymerase has been sequenced).

APPENDIX 1: Manuscripts Resulting from Grant N00014-87-K-0378

Copies of manuscripts published and submitted resulting from Grant ONR N00014-87-K-0378 have been submitted August 20, 1989 as Appendix I. The list submitted in 1989 is as follows:

Davis, J.T., Palmer, M.A.J., Peoples, O.P., Masamune, S., Sinskey, A.J. and Walsh, C.T. 1989. Biosynthetic thiolases from Zoogloea ramigera and Alcaligenes eutrophus: The inactivation with 5,5'-dithiobis(2-nitrobenzoate) and diethyl azodicarboxylate. Submitted to Biochemistry.

Differding, E., Gamboni, R., Williams, S.F., Palmer, M.A.J., Peoples, O.P., Masamune, S., Sinskey, A.J. and Walsh, C.T. 1989. Bio-Claisen condensation catalyzed by thiolase from Zoogloea ramigera: Inhibition with 3-pentynoyl-pantetheine-11-pivalate. Submitted to Biochemistry.

Jamas, S., Chen, S.-C.J., von der Osten, C.H., Sinskey, A.J. and Rha, C.K. 1989. Spectral analysis of glucan produced by wild-type and mutant Saccharomyces cerevisiae. Submitted to Carbohydrate Polymers.

Palmer, M.A.J., Gamboni, R., Williams, S.F., Peoples, O.P., Masamune, S., Sinskey, A.J. and Walsh, C.T. 1989. Bio-Claisen condensation catalyzed by thiolase from Zoogloea ramigera. Inactivation of acryl-S-pantetheine-11-pivalate and characterization of the Gly-378 mutant thiolase enzyme. Submitted to Biochemistry.

Peoples, O.P. and Sinskey, A.J. 1987. Polyhydroxybutyrate (PHB): A model system for biopolymer engineering. Prog. Biotechnol. 3:51-56.

Peoples, O.P. and Sinskey, A.J. 1989a. Fine structural analysis of the Zoogloea ramigera phbA-phbB locus encoding ß-ketothiolase and acetoacetyl-coa reductase: nucleoside sequence of phbB. Molecular Microbiology 3:349-357.

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Peoples, O.P. and Sinskey, A.J. 1989c. Poly-ß-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Characterization of the genes encoding ß-ketothiolase and acetoacetyl-coa reductase. J. Biol. Chem. 264:

APPENDIX 2: Patents Filed and Pending based on support for Grant ONR N00014-87-K-0378

Peoples, O.P. and Sinskey, A.J. "Method for Producing Novel Polyester Biopolymers." MIT-4403, Filed 1987.

Sinskey, A.J., Easson, D.D., Jr., Peoples, O.P. and Rha, C.K. "Methods to Control and Produce Novel Biopolymers." MIT-4010(2). Filed October 28, 1986.

Easson, D.D., Jr., Sinskey, A.J. and Peoples, O.P. "Methods for Altering Surface Change of Microorganisms." MIT-4510.

These patent applications have all been submitted to ONR previously.

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12 PERSONAL AUTHOR(S)

Peoples, Oliver P. and Sinskey, Anthony J.

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Sponsored by Molecular Biology Program

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Biopolymer engineering; polyhydroxybutyrate genetic engineering; Alcaligene eutrophus; Zoogloea ramigera; Pseudomonas oleovarans

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The overall objective of this proposal is to study the genetics and enzymology of polyhydroxybutyrate (PHB) biosynthesis at the molecular level in order to provide strategies for the development of new PHB-like biopolymers. Specific aims are to (1) control the chain length of the PHB polymers produced in fermentation processes through genetic manipulation; (2) incorporate different monomers into PHB producing co-polymers with different physical properties, and (3) examine the physical/ rheological properties of these new biopolymers in order to develop criteria at the molecular level.

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(phbC) in a 1.8 Kb fragment upstream from the phbA-phbB genes encoding β-ketothiolase and NADP-specific acetoacetyl-CoA reductase. The complete nucleotide sequence of this fragment was determined. A single long open reading frame extends from the ATG or nucleotide 842 to the TGA stop codon at nucleotide 2608 encoding a polypeptide of 589 amino acids with an M_r 63,940. The phbC structural gene terminates 85 bp upstream from the start of the phbA gene. Hence in A. eutrophus the PHB biosynthetic enzymes are encoded by the phbC-phbA-phbB genes organized as illustrated on Figure 1.

Introduction of all three genes into E. coli results in the synthesis of significant levels of PHB (plasmid pAeT41, Table 1), however, expression of phbC alone does not result in PHB production (plasmid pAeT42, Table 1). Preliminary results from the analysis of phbC-lacZ fusion plasmids indicate that in E. coli, phbC is constitutively expressed even although PHB biosynthesis proceeds only after depletion of nitrogen. This analysis is now being performed in A. eutrophus. Work is currently underway to investigate the potential application of increasing the level of expression of each of the three genes on the kinetics of PHB production by A. eutrophus. It will be of particular interest to determine if increasing phbC expression affects the molecular weight of the PHB produced. For mechanistic studies on the polymerase, the phbC structural gene has been inserted into a tac promoter vector to overproduce the enzyme.

GENES INVOLVED IN THE BIOSYNTHESIS OF POLYHYDROXYALKANOATE (PHA) POLYESTERS IN *PSEUDOMONAS OLEOVARANS*

In 1983, de Smet et al. (de Smet, M.J., Eggink, G., Witholt, B., Kingma, J. and Wynberg, H., J. Bacteriol. 154:870-878) identified a polymer produced by *Pseudomonas oleovarans* TF4-1L (ATCC 29347) as poly-ß-hydroxyoctanoate. Subsequent studies showed that *P. oleovarans* could produce a range of PHA biopolymers depending on the carbon source used, i.e., n-alkanes and 1-alkenes (Lageveen et al., 1988; Applied and Environmental Microbiology 54:2924-2932) or fatty acids (Brandl et al., 1988; Applied & Environmental Microbiology 54:1977-1982). The pathway proposed involves the conversion of the alkanes/alkenes to the fatty acid which then enters the fatty acid ß-oxidation pathway resulting in the formation of the D isomer of the ß-hydroxyacyl-CoA which is incorporated into the polymer by PHA polymerase. *P. oleovarans* has been shown not to incorporate ß-hydroxybutyrate indicating that 1) it does not possess the thiolase/reductase enzymes, or 2) the PHA polymerase cannot use ß-hydroxybutyrate as a substrate. The broad range of substrates used by the *P. oleovarans* PHA polymerase make the gene encoding this enzyme particularly interesting for biopolymer engineering of polyesters.

We followed the approach used for isolating the *A. eutrophus* ß-ketothiolase and NADP-specific acetoacetyl-CoA reductase using the *Z. ramigera* ß-ketothiolase gene as a DNA hybridization probe (Peoples and Sinskey, 1989a, J. Biol. Chem., in press) to isolate the *P. oleovarans* PHA polymerase gene. Southern DNA hybridization of *P. oleovarans* chromosomal DNA identified a 6 Kb EcoR1 restriction fragment with strong homology to the *A. eutrophus* PHB polymerase gene (phbC, Peoples and Sinskey, 1989b. J. Biol. Chem., in press).

The 6 Kb EcoR1 fragment was cloned in the *E. coli* plasmid vector, pUC18, by standard procedures to give plasmid pPO23 (Figure 1). The region which hybridized to the *A. eutrophus* phbC gene is located as indicated on Figure 1. Nucleotide sequence analysis of the complete 6 Kb fragment identified three potential protein coding regions (open reading frames, ORF1, ORF2 and ORF3, indicated on Figure 1). ORF1 begins at the ATG initiation codon nucleotide 554 and ends at the TGA stop codon nucleotide 2231 (Figure 2). This open reading frame is contained in the region of the pPO23 insert which hybridizes with the *A. eutrophus* phbC gene. ORF1 encodes a polypeptide of 562 amino acids with an M_r = 60,000. A comparison of the protein sequence predicted by translation of ORF1 with the amino acid sequence of the *A. eutrophus* PHB polymerase (Peoples and Sinskey, 1989b) using the program ALIGN revealed 52% identity between the two proteins. These data identify ORF1 as the *P. oleovarans* PHA polymerase gene. ORF2 begins at the ATG position 2297 and ends at the TAA position 3146 (Figure 2). ORF2 begins at the ATG position 3217 and ends at the TGA position 4948 (Figure 2). Both of these reading frames encode proteins, however, the identity of these proteins remains unclear. At this stage, we can only say that ORF2 and ORF3 are probably co-transcribed with the PHA polymerase gene (ORF1) and are probably involved in PHA biosynthesis.

WORK PLAN (Year 03)

The regulation of PHB metabolism will be investigated using phbC-lacZ fusion genes to monitor phbC expression in A. eutrophus. Antibodies to the PHB polymerase will be used to study the effect on the location of the polymerase enzyme under various physiological conditions. With respect to both the phbC gene product and the PHA polymerase from P. oleovarans, we

intend to overproduce and purify both enzymes for substrate specificity and mechanistic analysis. Once completed, these studies will enable us to develop rational strategies for manipulating the PHB/PHA biosynthetic pathways for increased productivity and the development of novel biopolymers.

PUBLICATIONS

Peoples, O.P. and Sinskey, A.J. 1989. Identification of the poly-ß-hydroxybutyrate (phbC) locus in *Alcaligenes eutrophus* H16. J. Biol. Chem., in press.

Peoples, O.P. and Sinskey, A.J. 1989. Poly-(ß)-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16: Analysis of the genes encoding ß-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem., in press.

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Figure 1. Organization of the A. eutrophus phbC-phbA-phbB genes.

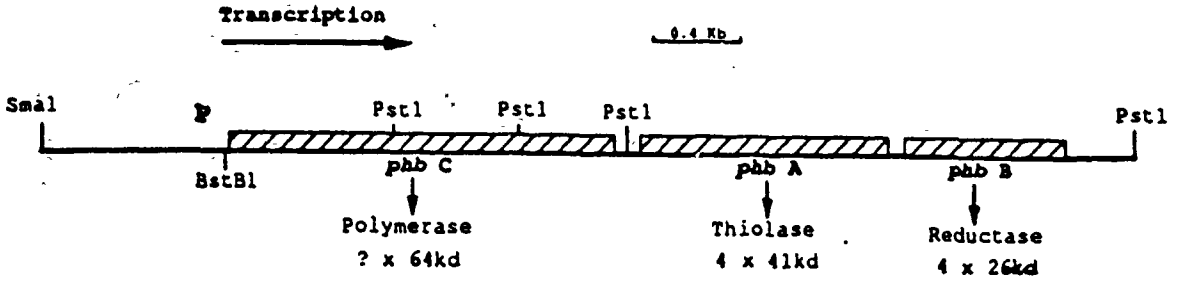
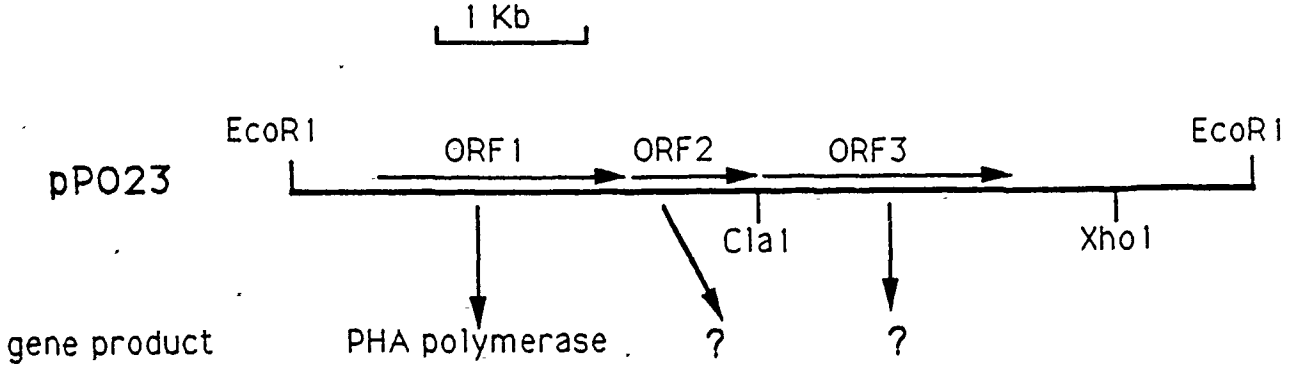


Table 1. Expression of the A. eutrophus phbC-phbA-phbB genes in E. coli

Plasmid	Thiolase U/mg protein	Reductase U/mg protein	Polymerase cpm/min/mg protein	Poly-β-hydroxybutyrate mg/mg protein
pUC18	0.5	ND	ND	0.015
pAeT41	59.0	2.5	2.4×10^4	2.977
pAeT42	0.9	ND	0.02×10^4	0.011

ND: no detectable activity

Figure 2. Organization of the P. oleovarans PHA polymerase locus



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PROGRESS REPORT ON CONTRACT N00014-87-K-0378

PRINCIPAL INVESTIGATOR: Anthony J. Sinskey

CO-INVESTIGATORS: Oliver P. Peoples, ChoKyun Rha

CONTRACTOR: M.I.T.

CONTRACT DATE: March 2, 1987

PERIOD COVERED: July 1, 1987 - June 30, 1988

RESEARCH OBJECTIVE

The overall objective of this proposal is to study the genetics and enzymology of polyhydroxybutyrate (PHB) biosynthesis at the molecular level in order to provide strategies for the development of new PHB-like biopolymers. Specific aims are to 1) control the chain length of the PHB polymers produced in fermentation processes through genetic manipulation, 2) incorporate different monomers into PHB producing co-polymers with different physical properties, and 3) examine the physical/rheological properties of these new biopolymers in order to develop design criteria at the molecular level.

PROGRESS

The characterization of the PHB-biosynthetic β-ketothiolase and acetoacetyl-CoA reductase enzymes encoded by the A. eutrophus phbA and phbB genes is almost complete. The thiolase can utilize C_4, C_5 and C_6 β-ketoacyl-CoA substrates with decreasing efficiency (100%, 40%, 5%). The reductase is strictly specific for the D-isomer of β-hydroxybutyryl-CoA and for NADPH as the cofactor. The reductase can also utilize the C_4, C_5, and C_6 β-ketoacyl-CoA substrates. Potentially these enzymes could synthesize C_5 and C_6 substrates for the PHB polymerase. In fact it is very likely that this is

the route for incorporation of D-β-hydroxyvalerate (C_5) to form the PHB-PHV copolymer produced by A. eutrophus when grown on propionate (BIOPOL).

The isolation and characterization of the genes encoding PHB synthase is currently the major focus of our molecular biology studies. Progress towards this goal has been made as follows. A Tn5 mutant library of A. eutrophus has been screened for PHB^- mutants by analyzing colonies on minimal fructose plates limited for nitrogen. 32 mutants (#1-#32) defective or deficient in PHB production as judged by their opaque appearance (PHB^+ colonies remain white) were selected at random for further study. A combination of DNA hybridization and biochemical analysis was used to characterize the mutant strains. From DNA hybridization studies it became clear that there were only three different Tn5 insertion mutants of which we had selected multiple copies of class "A" mutants (typified by strain #2) multiple copies of "class B" mutants (typified by strain #3) and a single class "C" mutant, strain #19. Moreover, for class "A" and "B" mutants we were able to identify the location of the Tn5 insertions approximately 1.2 kb and 1.6 kb upstream from the thiolase and reductase genes, respectively (Figure 1). For strain #19, the Tn5 has inserted elsewhere on the chromosome. Biochemical analysis of each mutant strain demonstrated that strains #2, #3 and #19 produced no PHB and had no PHB polymerase activity (Table 1). In each of the mutant strains, the level of β-ketothiolase and acetoacetyl-CoA reductase enzyme activities were reduced by around 50-70%.

The next step was to construct a series of plasmids containing restriction fragments from plasmid pAeT29 (Figure 1) in the broad host range vector pLAFR3 (Figure 2). Introduction of each of the plasmids into each of the mutant strains resulted in the complementation of the PHB^- phenotype. Biochemical analysis of the complemented strains confirmed the production of

PHB and PHB polymerase activity. From these experiments we conclude that the PHB polymerase gene (phbC) is located upstream from phbA and phbB in a 1.8 kb fragment (Figure 3). More recently, we have demonstrated that the fragment when introduced into E. coli on the pUC18 vector (plasmid pAeT42) results in the production of PHB in this org... These studies are now being performed in greater detail using a vari... substrates to determine the potential for using recombinant E. coli as a ... production strain.

Pseudomonas oleovarans TF4L has been shown to produce a range of PHA biopolymers by incorporating C_6, ... and C_{ℓ} units. We therefore used the A. eutrophus phbC gene as a hybridization probe to isolate the P. oleovarans phbC locus. The nucleotide sequence of this gene is currently being determined for comparison with the A. eutrophus gene.

Work Plan (Year 02: 7/1/88 - 6/30/89)

We are currently completing the nucleotide sequences of phbC from both A. eutrophus and P. oleovarans TF4L. The fact that introducing the phbC gene into E. coli leads to the production of PHB in this strain indicates that PHB polymerase can be functionally expressed in this bacteria. We will over-produce the enzyme from both A. eutrophus and P. oleovarans for purification and substrate specificity analysis. Clearly, we can now begin to unravel the mechanisms regulating the expression of the phbA,B,C genes in A. eutrophus and also investigate the potential for constructing new production strains by genetic manipulation.

PUBLICATION AND REPORTS

1. Papers

Peoples, O.P. and Sinskey, A.J. Identification of the poly-β-hydroxybutyrate (phb C) locus in Alcaligenes eutrophus H16. Manuscript in preparation.

Peoples, O.P. and Sinskey, A.J. Poly-β-hydroxybutyrate biosynthesis in Zoogloea ramigera. The genes encoding β-ketothiolase and acetoacetyl-CoA reductase are organized as an operon. Submitted to Molecular Microbiology.

Peoples, O.P. and Sinskey, A.J. Poly-β-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16: Analysis of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. Submitted to Journal of Biological Chemistry.

Peoples, O.P. and Sinskey, A.J. 1988. Expression of the Zoogloea ramigera biosynthetic thiolase gene in E. coli. Submitted to J. Bacteriol.

2. Presentations

Peoples, O.P. and Sinskey, A.J. 1988. Genetics and Molecular Biology Studies on PHB Biosynthesis. Presented at the Symposium on Biocatalysis and Biomimetics: Polymers in Biotechnology. 3rd Chemical Congress of North America, Toronto, Canada, June 5-10, 1988.

Table 1

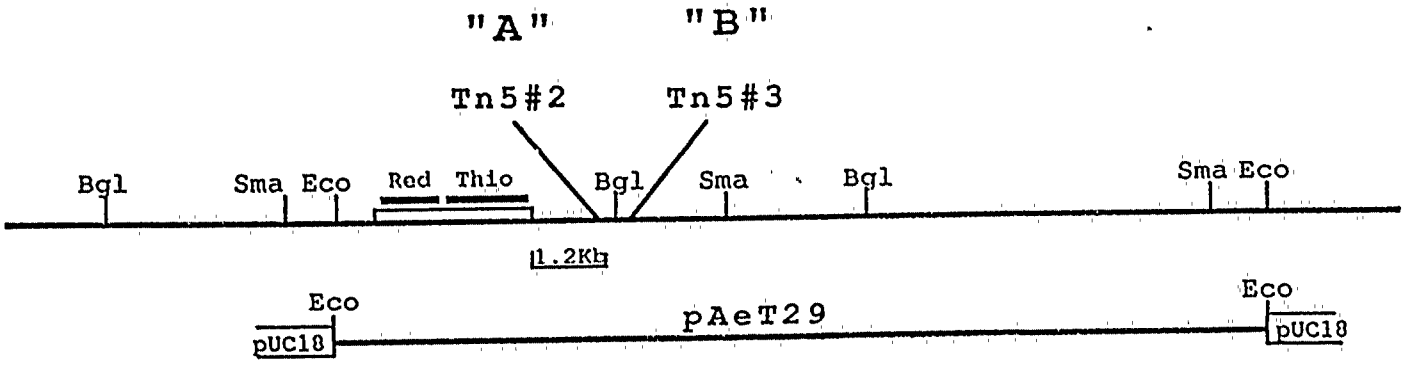
Analysis of PHB-Negative *A. eutrophus* Strains

Strain	PHB[1]	Thiolase[2]	Reductase[2]	Polymerase[3]
H16	0.23	8.9	1.6	8×10^3
Tn5#2	<0.01	4.0	0.5	–
Tn5#3	<0.01	3.4	0.5	–
Tn5#19	<0.01	3.2	0.4	–

[1] mg/mg of protein
[2] units/mg of protein
[3] cpm/min/mg of protein

Figure 1

Location of Chromosomal Tn5 insertions



"C"

Tn5#19 located elsewhere in the genome

Figure 2

Plasmid Constructions in pLAFR3
for Complementation of PHB Mutants

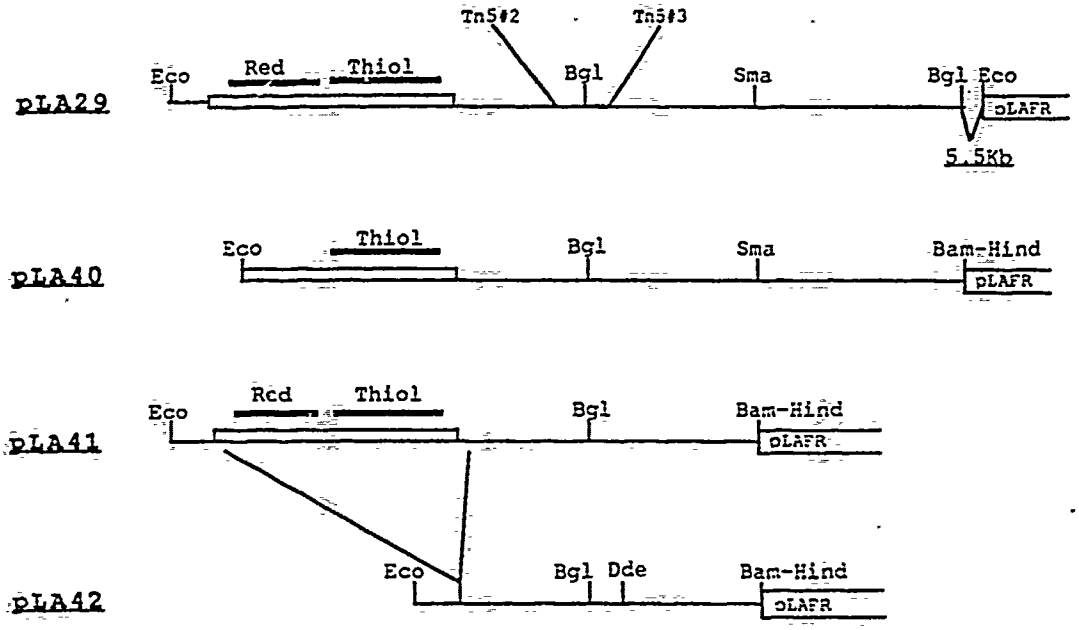
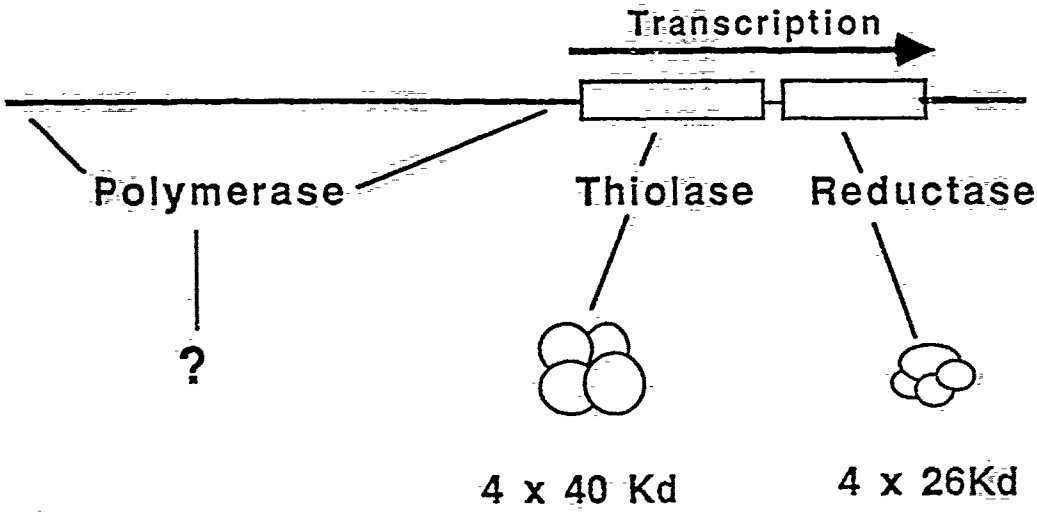


Figure 3

A.eutrophus

phb Genes are Organised as a Potential Operon



Synthesis of (D-) B-Hydroxy-CoA Esters

C_4 — C_6

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CONTRACTOR: M.I.T.

CONTRACT DATE: March 2, 1987

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RESEARCH OBJECTIVE

The overall objective of this proposal is to study the genetics and enzymology of polyhydroxybutyrate (PHB) biosynthesis at the molecular level in order to provide strategies for the development of new PHB-like biopolymers. Specific aims are to 1) control the chain length of the PHB polymers produced in fermentation processes through genetic manipulation, 2) incorporate different monomers into PHB producing co-polymers with different physical properties, and 3) examine the physical/rheological properties of these new biopolymers in order to develop design criteria at the molecular level.

PROGRESS

During the period covered by this report, we have focused on completing the isolation and characterization of the genes and enzymes of the PHB biosynthetic pathway from both Zoogloea ramigera and Alcaligenes eutrophus. In addition, work on purifying and examining the physical properties of polyhydroxyalkanoates from A. eutrophus and Pseudomonas oleovorans is currently underway. Progress has been made in a number of areas of our genetic studies.

Site-directed mutagenesis studies on the Z. ramigera β-ketothiolase to identify the catalytic amino acids and substrate binding site has been carried

out. Four specific mutants have been constructed and are currently being characterized: mutant #1, residue Cys_{89} has been changed to Ser_{89}; mutant #2 residue His_{124} has been changed to Asn_{124}; mutant #3, residue Cys_{125} has been chnaged to Ser_{124}; and mutant #4, residue His_{347} changed to Asn_{347}.

Overproduction in E. coli of the Z. ramigera acetoacetyl-CoA reductase and both the A. eutrophus β-ketothiolase and acetoacetyl-CoA reductase has been accomplished essentially as outlined in the proposal. A yield of 25-30% total cell protein is obtained when E. coli JM105 cells containing the plasmid pAT5 (A. eutrophus β-ketothiolase gene) are induced by IPTG. This is more or less identical to the result with the thiolase from Z. ramigera and this enzyme is also readily purified in 150 mg/l cell quantities. Both reductases are overproduced at a level of 7-10% total cell protein in E. coli containing the plasmids pZR14 (Z. ramigera reductase gene) and pAR1 (A. eutrophus reductase gene). A two step purification has also been developed. All of these enzymes are currently being analyzed for potential alternate substrates.

The isolation and characterization of the genes encoding PHB synthase is the major focus of our molecular biology studies. Progress towards this goal has been made as follows. PHB granules have been isolated from Z. ramigera and SDS-polyacrylamide gel electrophoresis of the protein extracted from the granules indicates three major polypeptides of around M_r 43,000. A Tn5 mutant library of A. eutrophus has been screened for PHB^- mutants by analyzing colonies on minimal fructose plates limited for nitrogen. 32 mutants defective or defficient in PHB production as judged by their opaque appearance (PHB^+ colonies remain white). We are currently analyzing these mutant strains both by enzyme assay for β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase as well as by Southern blot hybridization.

WORK PLAN (YEAR 01; 7/1/87 - 7/1/88)

Work is currently underway to determine the substrate specificities of the β-ketothiolase and acetoacetyl-CoA reductase enzymes, overproduced in and purified from E. coli. These studies will be carried out together with mechanistic enzymology will lead to the identification of potential alternate substrates for PHB synthesis. Our major focus will remain on the isolation and characterization of the gene(s) encoding PHB synthase. To this end we will continue the analysis of the PHB$^-$ mutants of A. eutrophus so far identified eventually leading to complementation studies to clone the gene(s). We will then sequence, overproduce and purify the synthase for substrate specificity and kinetic analysis. Work on the physical/chemical properties of polyhydroxyalkanoates will focus on copolymers purified from A. eutrophus grown on propionate and P. oleovorans grown on octane.

INVENTIONS

"Method for producing novel polyester biopolymers", Peoples and Sinskey: MIT 4403 U.S.S.N 067,695.

PUBLICATIONS AND REPORTS

1. Papers

a. High level expression of the Zoogloea ramigera biosynthetic thiolase gene in E. coli. J. Bacteriol. Submitted.

b. Easson, D.D. Jr., Sinskey, A.J. and Peoples, O.P. 1987. Isolation of Zoogloea ramigera I-16-M exopolysaccharide biosynthetic genes and evidence for instability within this region. J. Bacteriol. In Press.

Two additional manuscripts are currently being prepared, the first concerns the identification and analysis of the Z. ramigera acetoacetyl-CoA reductase gene and the second the isolation and characterization of the A. eutrophus β-ketothiolase acetoacetyl-CoA reductase genes.

2. Presentations

Peoples, O.P. and Sinskey, A.J. 1987. Polyhydroxybutyrate (PHB): A Model System for Biopolymer Engineering. Presented at the Symposium on Modification and Application of Industrial Polysaccharides. 193rd ACS National Meeting, Denver, Colorado, April 5-10, 1987.

Peoples, O.P. and Sinskey, A.J. 1987. Polyhydroxybutyrate (PHB): A Model System for Biopolymer Engineering. Presented at the "Materials Biotechnology Symposium", Natick Research, Development and Engineering Center. Natick, MA, June 23-24, 1987.

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